Developmental Biology **249,** 16–29 (2002)
doi:10.1006/dbio.2002.0751

The Mouse Kreisler (*Krml1/MafB*) Segmentation Gene Is Required for Differentiation of Glomerular Visceral Epithelial Cells

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Molecular components of the glomerular filtration mechanism play critical roles in renal diseases. Many of these components are produced during the final stages of differentiation of glomerular visceral epithelial cells, also known as podocytes. While basic domain leucine zipper (bZip) transcription factors of the Maf subfamily have been implicated in cellular differentiation processes, *Kreisler (Krml1/MafB),* the gene affected in the mouse *kreisler (kr)* mutation, is known for its role in hindbrain patterning. Here we show that mice homozygous for the kr^{enu} mutation develop renal disease and that *Kreisler* is essential for cellular differentiation of podocytes. Consistent with abnormal podocyte differentiation, kr^{enu} homozygotes show proteinuria, and fusion and effacement of podocyte foot processes, which are also observed in the nephrotic syndrome. Kreisler acts during the final stages of glomerular development—the transition between the capillary loop and mature stages—and downstream of the Pod1 basic domain helix–loop–helix transcription factor. The levels of Podocin, the gene mutated in autosomal recessive steroid-resistant nephrotic syndrome (NPHS2), and Nephrin, the gene mutated in congenital nephrotic syndrome of the Finnish type (NPHS1), are slightly reduced in kr^{enu}/kr^{enu} podocytes. However, these observations alone are unlikely to account for the aberrant podocyte foot process formation. Thus, Kreisler must regulate other unknown genes required for podocyte function and with possible roles in kidney disease. **© 2002 Elsevier Science (USA)**

***Key Words:* *Kreisler (Krml1/MafB)*; *Pod1 (epicardin/capsulin)*; podocyte; kidney disease; cellular differentiation; proteinuria.**

INTRODUCTION

In the kidney, the glomerular filtration apparatus consists of several highly differentiated cell types: mesangial cells, podocytes, and the specialized fenestrated capillary endothelium. Both podocytes and endothelial cells produce the glomerular basement membrane (GBM), which surrounds the mesangial cells and the capillaries (Lehtonen *et al.*, 2000; Li *et al.*, 2000). The mesangial cells provide structural support to the capillaries located immediately adjacent to them. The podocytes, or visceral glomerular epithelial cells, produce long slender cellular processes, known as pedicles or foot processes, that wrap around the GBM encased capillary and together with the GBM form a blood filtration barrier that maintains selective permeability to proteins.

Damage to podocytes causes renal diseases, such as minimal change nephrosis (MCNS) or focal segmental glomerulosclerosis (FSGS). Aside from diabetes, FSGS is currently the main cause of renal insufficiency in humans. Kidney disease is often progressive. At the onset of renal pathogenesis, the GBM thickens and podocytes assume ultrastructural characteristics of less mature glomeruli. In glomerulosclerosis, mesangial cells and an apparently thickened GBM expand and begin to displace damaged

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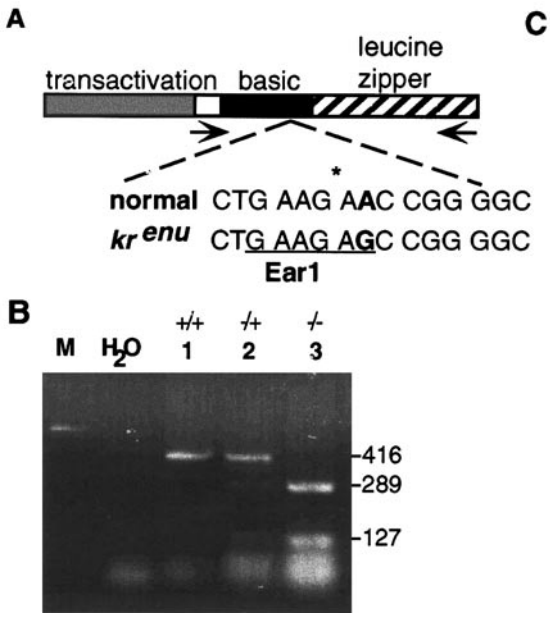
0012-1606/02 $35.00



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Genotypes of Embryos and Mice from *kr enu* intercrosses

	+/+	+/kr enu	kr enu/kr enu
E8.5	14	43	31
E9.5	24	50	23
E10.5	2	7	1
E11.5	12	31	29
E12.5	0	3	2
E13.5	4	20	8
E14.5	5	8	4
E15.5	10	24	6
E16.5	3	5	2
birth	1	7	4
3wks	15	39	0

FIG. 1. Mice homozygous for the kr^{enu} mutation die as neonates. (A) A schematic diagram shows the novel *Ear*I recognition site generated by the kr^{enu} mutation in the DNA-binding domain of *Kreisler* and the location of primers used to amplify this region. (B) *Ear*I digestion of the PCR product from normal *Kreisler* results in a 416-bp undigestable fragment present in +/+ and +/kr^{enu} mice, while *Ear*I digestion of the PCR fragment generated from kr^{enu} DNA yields 289- and 127-bp fragments present in kr^{enu} heterozygous and homozygous animals. (C) The table shows the genotypes of complete litters from kr^{enu}/+ intercrosses at various stages of development. No decrease in viability of kr^{enu}/kr^{enu} embryos was observed throughout embryogenesis or at birth. However, within 24 h after birth, all kr^{enu}/kr^{enu} mice have died. No weanling or adult kr^{enu} homozygotes have ever been recovered. Only complete litters harvested are shown here. Additional kr^{enu} homozygotes and normal littermates were harvested at 14.5 and 15.5 dpc and as neonates for the experiments performed here.

podocytes, which have a dedifferentiated appearance, and ultimately kidney failure results. Such dedifferentiation of podocytes and loss of the molecular components of the filtration apparatus are associated with genetic renal diseases (Kriz *et al.*, 1994; Pagtalunan *et al.*, 1997). Recent advances in human genetics further underscore the importance of the podocyte. Mutations in genes that encode components of the specialized podocyte cytoskeleton can cause genetic renal disease. In mice and humans, the expression of these genes is highly podocyte-specific and must be regulated by podocyte-specific differentiation factors. However, only a few transcription factors with roles in glomerular development have been identified, and even fewer are known to regulate podocyte differentiation specifically (Lechner and Dressler, 1997). The identification of regulatory molecules that could reactivate proper expression of the key structural components of the podocytes, the filtering pedicles, and the GBM would be necessary to resuscitate injured podocytes or induce development of new ones.

Members of the Maf subfamily of basic domain leucine zipper transcription factors play key roles in cellular differentiation (Blank and Andrews, 1997). Loss-of-function mutations in mouse c-Maf and MafG interrupt terminal differentiation of lens fiber cells and megakaryocytes, respectively (Kim *et al.*, 1999; Ring *et al.*, 2000; Shavit *et al.*, 1998). Furthermore, potential binding sites for Maf protein homodimers or heterodimers have been found in the promoters of important cell type-specific genes, such as β-globin, heme oxygenase, interleukin-4, and crystallins, and expression of these genes can be regulated by specific Maf proteins in transient transfection assays (Inamdar *et al.*, 1996). In contrast to other Maf family members, *Kreisler* (*Krml1/MafB*; known as MafB according to mouse nomenclature) is known for its role in embryonic patterning rather than any functions in cellular differentiation. *Kreisler* was originally identified as the gene affected in the *kreisler* (*kr*) mouse mutation, in which a primary defect in embryonic hindbrain patterning causes circling behavior and deafness in *kr/kr* adults (Cordes and Barsh, 1994; Deol, 1964). The embryonic hindbrain is transiently subdivided into seven to eight segments, known as rhombomeres. *Kreisler* is expressed transiently in the fifth and sixth rhombomeres during hindbrain segmentation (Cordes and Barsh, 1994). In animals with mutations in *Kreisler*, the fifth and sixth rhombomeres do not form properly and rhombomere-specific expression of *Hox* genes is disrupted in r5 and r6 (Cordes and Barsh, 1994; Manzanares *et al.*, 1997, 1999; Moens *et al.*, 1998). In the mouse, there are two mutant alleles of *Kreisler*: the viable X-ray-induced *kr* allele and the

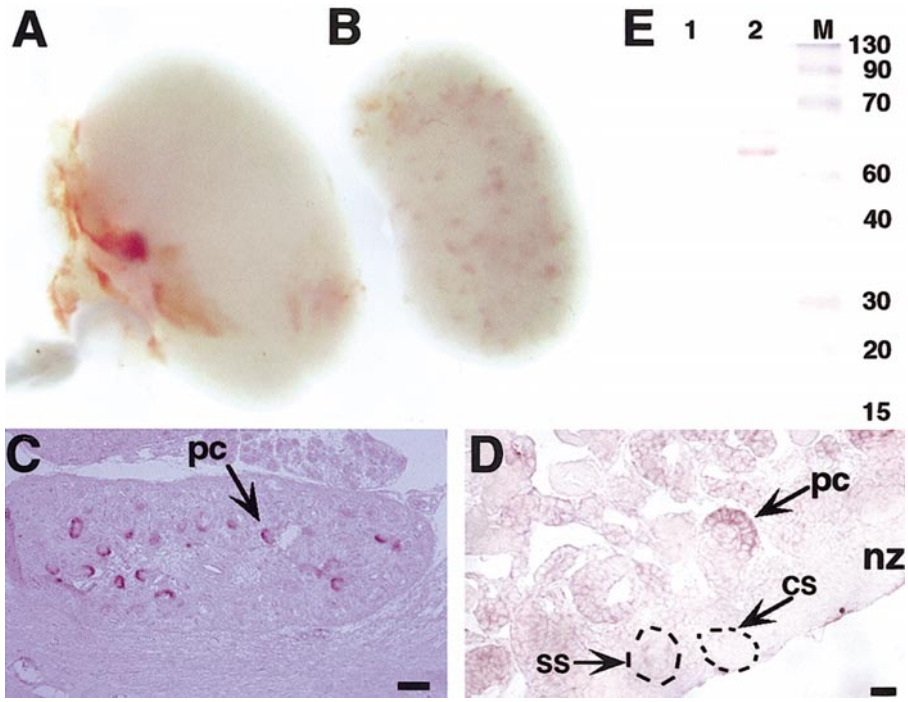


FIG. 2. Defects in appearance and function of neonatal kidneys from kr^{enu}/kr^{enu} mice. Unlike kidneys from $+/kr^{enu}$ neonates (A), those from kr^{enu}/kr^{enu} neonates (B) have a punctate cortical surface and slightly shrunken appearance. (C) *Kreisler* is expressed at high levels in podocytes of 14.5-dpc kidneys as detected by digoxigenin RNA *in situ* hybridization on sections. The scale bar represents 100 μm. (D) *Kreisler* continues to be expressed in podocytes (pc) of weanling mice as shown here by dideoxygenin RNA *in situ* hybridization on sections from a 3-week-old $kr^{enu}/+$ mouse. In the kidney, *Kreisler* expression could only be detected in podocytes, which in sections are organized in a characteristic crescent shape at the outer edge of glomeruli. *Kreisler* expression could not be detected in comma-shaped (cs)- and S-shaped-stage glomeruli (ss) located near the cortical surface in the nephrogenic zone (nz), but only in the podocytes of the more internally located capillary loop stage or mature glomeruli. The scale bar represents 20 μm. (E) kr^{enu}/kr^{enu} neonates exhibit proteinuria. In lane 1, no protein could be detected in 0.1 μl of urine from heterozygous neonates on a Coomassie blue-stained 10% polyacrylamide minigel. However, in lane 2, significant amounts of an ~67-kDa protein, which is consistent with the expected size of serum albumin, could be detected in 0.1 μl of urine from kr^{enu}/kr^{enu} neonates. Lane 3 shows protein markers of the sizes indicated in kDa.

lethal kr^{enu} allele, which we generated previously by chemical mutagenesis with ethylnitrosourea (ENU). The unexpected lethality of kr^{enu} homozygotes suggested that further analysis of the phenotypes of mice homozygous for the kr^{enu} allele would reveal additional essential functions of the *Kreisler* gene, possibly in cellular differentiation. Here we show that *Kreisler* is required specifically for podocyte differentiation.

MATERIALS AND METHODS

Mice and Genotyping

Fifteen CSJ/L male mice, which are homozygous for the *A* allele of the *agouti* coat color locus, were mutagenized with four weekly doses of 100 mg/kg ENU (Sigma). Upon recovery of fertility at 10–12 weeks postinjection, mutagenized males were bred to *a kr*/*A Kr* females, which had been maintained on the C3H/HeJ strain for more than 10 generations. We recovered 3 circling mice upon screening 597 weanling mice (Cordes and Barsh, 1994). Deafness was initially scored by lack of the Preyer's reflex. To determine whether the kr^{enu} mutation could be a new allele of *Kreisler*, we tested whether this mutation is linked to the agouti coat color locus. The original *A* kr^{enu}*/a kr* male recovered from this screen was bred to C57Bl/6J females; only the resulting *Agouti* female offspring had deaf, circling progeny when mated to *a kr/a kr* males. Only in the presence of the original *kr* allele do animals carrying the kr^{enu} mutation exhibit the characteristic circling and deafness. As described previously, we determined that the kr^{enu} mutation indeed represents a new allele of *Kr*. The original *A* kr^{enu}*/a kr* male recovered from this screen and subsequent *A* kr^{enu}*/a*+ heterozygotes have been crossed onto a C57Bl6/J background for more than 10 generations. For unambiguous determination of genotype, we developed a PCR assay that relies on an *Ear*I polymorphism generated by the A-to-G transition in the kr^{enu} allele. The basic domain leucine zipper region of *Kreisler* was amplified with primers 5′-GCAGAATAGGGAGTCTG and 5′-AAGGATCCGG-ACCGCTTCTCTGAGAC by 40 cycles of denaturing at 94°C, annealing at 54°C, and extending at 72°C for 1 min each. Digestion of the resulting 416-bp product with *Ear1*(New England Biolabs,

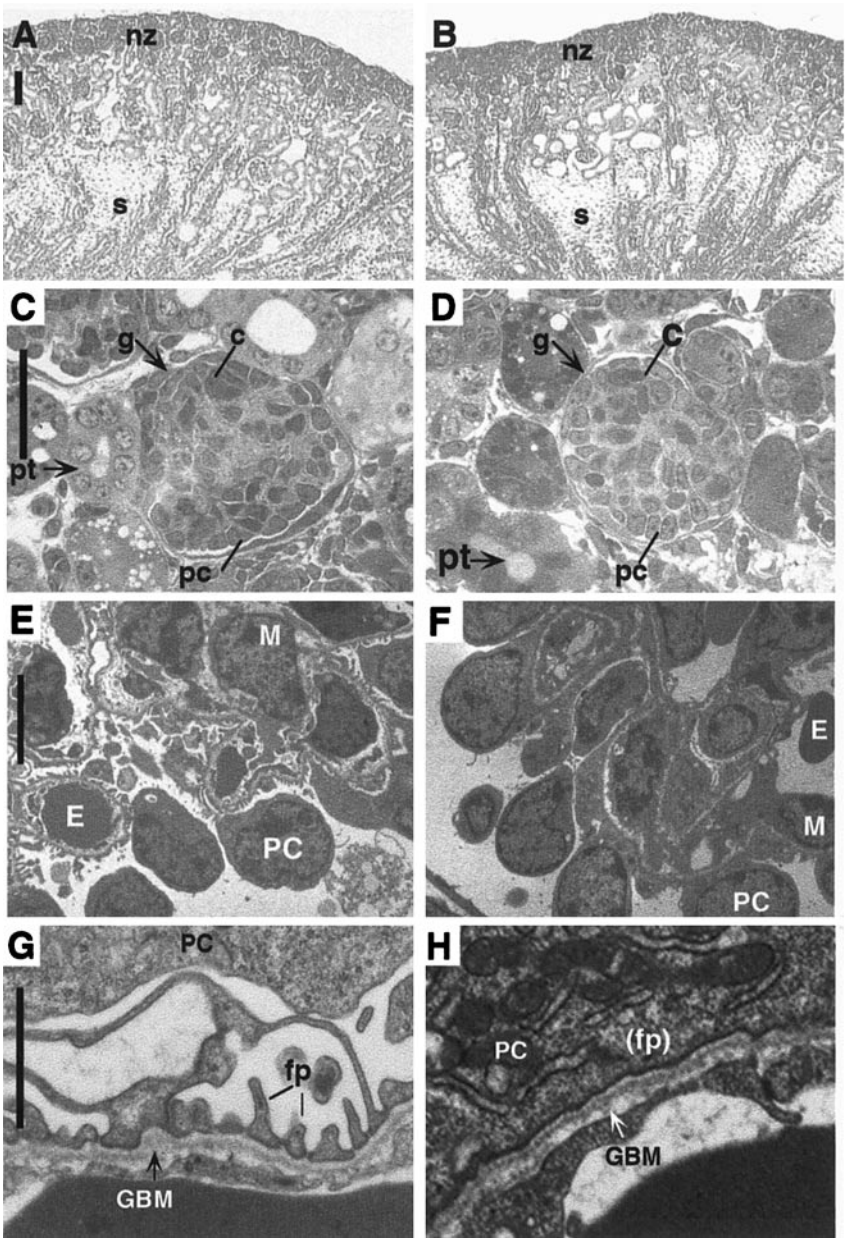


FIG. 3. kr^{enu}/kr^{enu} neonatal kidneys are histologically indistinguishable from +/+ and+/kr^{enu} kidneys. Sagittal sections of +/kr^{enu} (A) and kr^{enu}/kr^{enu} (B) neonatal kidneys stained with hematoxylin and eosin do not reveal any histological abnormalities The nephrogenic zone (nz), the birthplace of all glomeruli, is located on the outer rim of the cortex. Mature glomeruli are located more internally. Proximal (pt) and distal tubules are clearly visible just below the nephrogenic zone and are well-organized. The stroma (s) appears normal. Sagittal sections of mature glomeruli from +/kr^{enu} (C) and kr^{enu}/kr^{enu} (D) kidneys stained with toluidine blue show no abnormalities. Capillaries (c) and all glomerular cell types are present and appear morphologically normal. Podocytes (pc) are in normal positions and appear normal at this magnification. The scale bar in (A) indicates 60 μm, while that in (C) represents 20 μm. (B) is at the same magnification as (A), and (C) is at the same magnification as (D). (E–H) Lack of normal podocyte foot processes in kr^{enu}/kr^{enu} glomeruli. Electron microscopy reveals that all glomerular cell types—mesangial and endothelial cells as well as podocytes—are present in normal (E, G) and kr^{enu}/kr^{enu} (F, H) glomeruli. However, there are significant ultrastructural differences between glomeruli from kr^{enu}/+ (E, G) and kr^{enu}/kr^{enu} (F, H) neonates. Discrete foot processes can be seen in normal glomeruli (E, G). In (F,H), kr^{enu}/kr^{enu} mice podocyte foot processes (fp) are fused and do not interdigitate. Where these foot processes should be located is indicated with "fp" in parentheses. (E, F) Lower magnification views. The scale for these panels is shown in (E) and represents 2 μm. Higher magnification shows that, while podocyte foot processes are absent, the GBM of kr^{enu}/+ (G) and kr^{enu}/kr^{enu} (H) glomeruli appear indistinguishable. The scale for (G, H) is shown in (G) and represents 1 μm. E, erythrocytes; gbm, glomerular basement membrane; M, mesangial cells; pc, podocyte; fp, podocyte foot process.

Beverly, MA) results in 289- and 127-bp fragments diagnostic for the kr^{enu} allele. In addition to the whole litters collected and tabulated in Fig. 1C, 21 kr^{enu}/kr^{enu} mice and corresponding 26 +/+ and 27 $+/kr^{enu}$ littermates were collected for phenotypic analyses described.

Phenotypic Analysis

Urinanalysis. Urine was expressed from neonatal mice between 6–24 h after birth and analyzed by spotting onto Multistix 8 SG Reagent strips for Urinanalysis (Miles Inc., Diagnostics Division, Elkhart, IN). Then, 60 s after "spotting," the urine was analyzed for the presence of protein by comparison with the standardized chart. Urine from normal littermates ($kr^{enu}/+$ and +/+) and from *kr/kr* mice contained approximately 30 mg/dL of protein, while that from kr^{enu} homozygous neonates contained between 100 and 300 mg/dL. For protein analysis, 0.1 μl of urine from mutant and normal neonates was examined by electrophoresis on a 10% denaturing SDS–polyacrylamide minigel.

Analysis of Expression Patterns

Whole-mount RNA *in situ* analysis was performed as previously described by using sense and anti-sense probes generated from 1183 to 1411 of the *Kreisler* 3′UTR, and probes for *Hoxa3*, *Hoxb3*, and *Hoxd3* (Cordes and Barsh, 1994; Frohman *et al.*, 1993; Manley and Capecchi, 1998). Sense and anti-sense probes for *Nephrin* and *Pod1* were generated as previously described (Quaggin *et al.*, 1999; Wong *et al.*, 2000). Sense and anti-sense probes for *Podocin* (GenBank Accession No. AI552534) and *CD2AP* (GenBank Accession No. AA739155) were generated from ESTs obtained from the I.M.A.G.E. clone consortium (Genome Systems, Inc., Berkeley, MO). The CD2AP and Podocin anti-sense probes were generated by digestion of the plasmids with *Eco*RI and transcription with T3 RNA polymerase. To generate sense probes for CD2AP and Podocin, the EST clones were digested with *Not*I and subsequently transcribed with T7 RNA polymerase.

For digoxigenin RNA *in situ* hybridization on sections, embryos were fixed in 4% paraformaldehyde overnight and embedded in O.C.T. Tissue-Tek embedding medium (Sakura Finetechnical Co, Ltd., Tokyo, Japan). Then, 12-μm sections were cut with a Leica CM3050 cryostat. Hybridizations were performed essentially as described in Storm and Kingsley (1996).

For histological analyses, tissues and embryos were fixed in 4% paraformaldehyde on phosphate-buffered saline overnight and embedded in paraffin. Then, 8- to 12-μm sections were cut with a microtome (Leica) and stained with hematoxylin and eosin. For electron microscopy, tissues were fixed in 2% glutaraldehyde in phosphate-buffered saline, and transmission electron microscopy was performed by standard methods.

For immunohistochemistry, a rabbit anti-Kreisler antibody was raised against Maltose-binding protein fused to a full-length Kreisler protein, which has been used in previous binding studies. Immunohistochemistry using this anti-Kreisler antibody was performed on 12-μm cryosections, which had been postfixed in 4% paraformaldehyde, rinsed in PBS, and blocked in 10% goat serum. Sections were incubated overnight with primary antibody at a 1:5000 dilution at 4°C, rinsed three times in PBS, and incubated at room temperature for 1 h with Cy3-donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides were once again rinsed three times in PBS and examined microscopically. Immunohistochemistry with anti-Laminin β2, anti-Integrin α3, anti-Podocin, anti-Nephrin, and anti-PCNA (Chemicon International, Inc., Temecula, CA) antibodies was performed as previously described (Holzman *et al.*, 1999; Kreidberg *et al.*, 1996; Noakes *et al.*, 1995; Sasaki *et al.*, 2002; Schwarz *et al.*, 2001).

The RNA levels of *Podocin*, *Nephrin*, and *α Actinin 4* were determined by RNA slot blot analyses. Two separate experiments were performed on three independent sets of RNA samples. RNA levels were quantified by using the Scion Image suite of programs to analyze the resulting autoradiographs. Amounts were standardized relative to the level of GAPDH present in each sample.

RESULTS

Neonatal Lethality of kr^{enu}/kr^{enu} Homozygotes

The kr^{enu} mutation was identified in a noncomplementation mutagenesis screen by the circling behavior and deafness of kr/kr^{enu} mice. However, no circling deaf mice have ever been recovered from $kr^{enu}/+$ intercrosses (Cordes and Barsh, 1994). We used an *Ear*I restriction site, which was created by the A-to-G transition responsible for the kr^{enu} mutation, in a polymerase chain reaction-based assay to unambiguously identify kr^{enu}/kr^{enu} and $kr^{enu}/+$ mice (Figs. 1A and 1B). We observed the expected Mendelian ratios of +/+, $kr^{enu}/+$, and kr^{enu}/kr^{enu} animals at all stages of embryonic development and at birth. kr^{enu} heterozygotes appeared indistinguishable from normal animals (Fig. 1C). Even though many kr^{enu}/kr^{enu} neonates suckled initially, all died severely dehydrated within 24 h of birth.

Several observations suggested that hindbrain-independent developmental roles of *Kreisler* must be responsible for the lethality of kr^{enu}/kr^{enu} neonates. First, during later embryonic stages and throughout adulthood, *Kreisler* is expressed in a wide range of tissues in a cell type-specific manner. In the original viable *kr* mutation, a submicroscopic chromosomal inversion abrogates *Kreisler* expression in the fifth and sixth rhombomeres of the embryonic hindbrain. All other domains of *Kreisler* expression examined in 8.5- to 11.5-, 14.5-, and 15.5-dpc embryos and in adult tissues by RNA *in situ* and Northern hybridization were equivalent in normal and *kr/kr* animals (data not shown) (Eichmann *et al.*, 1997). Secondly, the defects in hindbrain patterning are less severe in kr^{enu}/kr^{enu} than in *kr/kr* embryos (V.S.S., A. Sing, L. Mar, F.J., and S.P.C., unpublished observations). Finally, we have shown previously that Kreisler activates transcription of *Hoxb3* in the fifth rhombomere directly (Manzanares *et al.*, 1997). Kreisler-dependent transcription of *Hoxb3* in the fifth rhombomere cannot be detected in either *kr/kr* or kr^{enu}/kr^{enu} embryos (S.P.C., unpublished observations; Frohman *et al.*, 1993). Thus, transcriptional activation that depends on the presence of active Kreisler homodimers appears to be abrogated or severely reduced in kr^{enu} homozygotes

Morphologic Abnormality of kr^{enu}/kr^{enu} Kidneys

We observed a variety of morphologic abnormalities, which include enlarged ventricles in the brain and abnor-

mal kidneys, that in concert would compromise neonatal viability of kr^{enu}/kr^{enu} neonates. Most strikingly, kr^{enu}/kr^{enu} neonates can be readily distinguished from normal and heterozygous littermates by the appearance of their kidneys (Figs. 2A and 2B). Kidneys from kr^{enu}/kr^{enu} homozygotes appear shrunken and have punctate cortical surfaces with obvious superficial areas of hemorrhaging (Fig. 2B). Within the kidney, *Kreisler* expression commences specifically in glomeruli at 14.5 dpc and is maintained in adults (Figs. 2C and 2D). The renal vesicle, from which the nephron arises, proceeds through four morphologically identifiable and defined developmental stages: the vesicle or comma-shaped body, the S-shaped body, the capillary loop, and the mature stages (Abrahamson, 1991). *Kreisler* mRNA is not detected in glomeruli at the comma- or S-shaped stages, which are located near the outer margin of the cortex in the nephrogenic zone (Figs. 2C and 2D) (Eichmann *et al.,* 1997; Imaki *et al.,* 2000). *Kreisler* is expressed exclusively in the podocytes of the more internally located capillary loop-stage and mature glomeruli. Because members of the Maf subfamily of bZip transcription factors, such as *Kreisler*, play key roles in cellular differentiation, we examined kidneys from kr^{enu} homozygotes further for possible defects in podocyte differentiation.

Renal Function in Neonatal kr^{enu}/kr^{enu} Mice

To further evaluate renal function in kr^{enu}/kr^{enu} mice, we carried out a series of clinical laboratory and histologic studies. A standard urinalysis revealed an absence of urinary sediment but the presence of proteinuria and occasional hemoglobin. Quantitative analysis showed proteinuria in the range of 100–300 mg/dL ($kr^{enu}/+$, *kr*/+, or *kr*/*kr* animals were 30 mg/dL). Because suckling and food intake is limited in kr^{enu}/kr^{enu} neonates, the protein load on the kidneys is relatively low and conceivably proteinuria would be more extreme if this protein load was increased. There are several possible causes for proteinuria and the type of protein released is indicative of the cause. The presence of high levels of albumin in the urine is diagnostic of glomerular proteinuria, while defects in the tubular epithelium lead to presence of small proteins. Finally, overload proteinuria results in the accumulation of immunoglobin chains in urine. Analysis of urine from neonates by polyacrylamide gel electrophoresis revealed the presence of high levels of an ~67-kDa protein, which is consistent with the size of serum albumin (~67 kDa) and is characteristic of a glomerular protein leak (Fig. 2E). Therefore, the kr^{enu} mutation must cause a defect within the glomerulus and possibly the podocytes themselves.

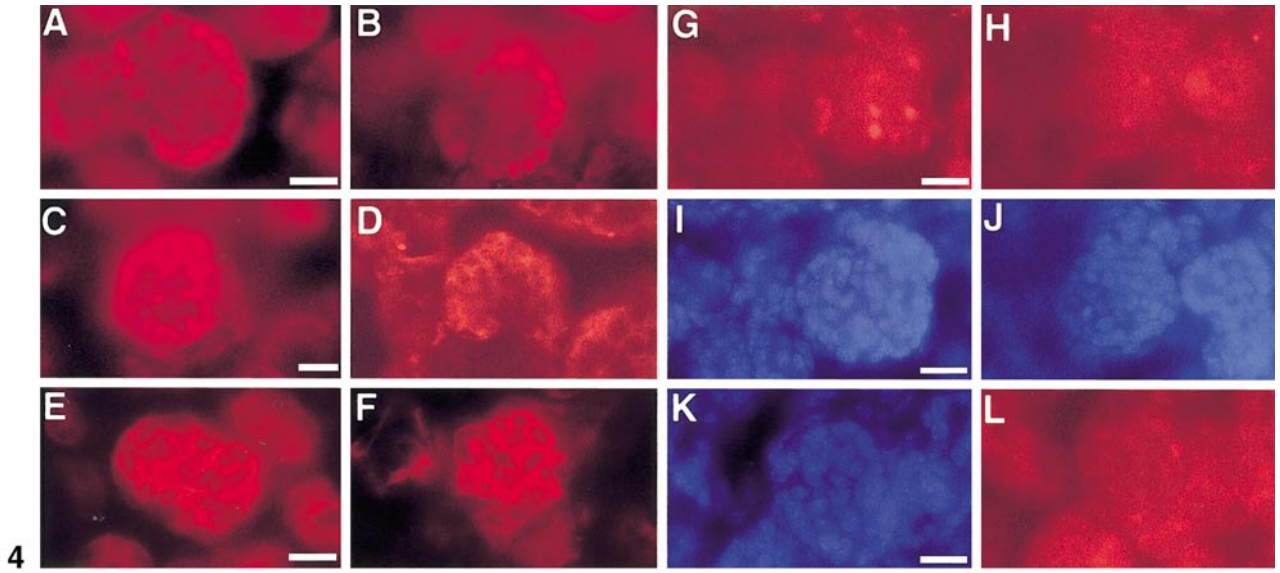
Podocyte Foot process Fusion in kr^{enu}/kr^{enu} Kidneys

Standard histologic analysis revealed no gross malformations at any stage of kidney development in kr^{enu}/kr^{enu} animals (Figs. 3A–3D). The nephrogenic zone, which is located near the external surface of the cortex and in which glomerular development begins, appeared normal in kr^{enu}/kr^{enu} animals. The stroma, proximal and distal tubules, ureter, and organization of nephrons in kr^{enu}/kr^{enu} kidneys appeared indistinguishable from those in normal kidneys (Figs. 3A and 3B). We had initially noticed the shrunken and spotted appearance of kidneys in kr^{enu} homozygotes. In mice homozygous for mutations in either PDGFβ or the PDGFβ receptor, a "spotted kidney" phenotype is seen and is caused by the absence of mesangial cells and abnormal vasculogenesis (Hellstrom *et al.,* 1999; Leveen *et al.,* 1994; Soriano, 1994). However, in kr^{enu}/kr^{enu} animals, mesangial cells and capillaries within glomeruli themselves are present and appear ultrastructurally normal (Fig. 3). Furthermore, no hemorrhaging or accumulation of blood is seen within glomeruli. Podocytes appear histologically normal as well (Figs. 3C and 3D). Finally, no defects in branching morphogenesis of the uretary bud epithelium of 13.5- or 15.5-dpc kidneys were detected by DBA–lectin staining (data not shown).

A glomerular protein leak, such as that observed in kr^{enu} homozygotes, is most often caused either by defects in the formation or maintenance of the glomerular basement membrane or by effacement of podocyte foot processes. Therefore, we examined neonatal kr^{enu}/kr^{enu} kidneys for ultrastructural defects by electron microscopy (Figs. 3E–3H). In the normal glomerulus, the podocyte and the GBM are responsible for maintaining the filtration slit structure that prevents protein escaping from the capillaries. The podocyte performs these functions by virtue of a complex array of interdigitating foot processes, or pedicles, that create the large intercellular surface area required for glomerular filtration and are located distant from the podocyte cell body. During the early capillary loop stage, when *Kreisler* expression is first observed, the epithelial cytoplasm appears as a continuous, uninterrupted mass without any foot processes (Abrahamson, 1991; Kriz *et al.*, 1994). As podocyte differentiation proceeds, the epithelium begins to interdigitate until the glomerular basement membrane is completely fringed by fully formed and distinct pedicles. Electron microscopy did not uncover any defects in the GBM. However, in kr^{enu} homozygotes, podocytes were present, but podocyte foot processes were absent or "fused" (Figs. 3E–3H). The epithelial cytoplasm is continuous and uninterrupted, and in essence, the podocytes in mature kr^{enu} glomeruli appear to be arrested in the capillary loop stage of normal development.

Podocytes Form Normally in Kidneys from kr^{enu} Homozygotes

At times, point mutations can compromise either protein stability or protein localization. To examine whether Kreisler protein was expressed at normal levels and within the nucleus of mutant podocytes, we performed immunofluorescence with an antibody raised against full-length mouse Kreisler protein (Figs. 4A and 4B). These immunofluorescence experiments detected nuclearly localized Kre-



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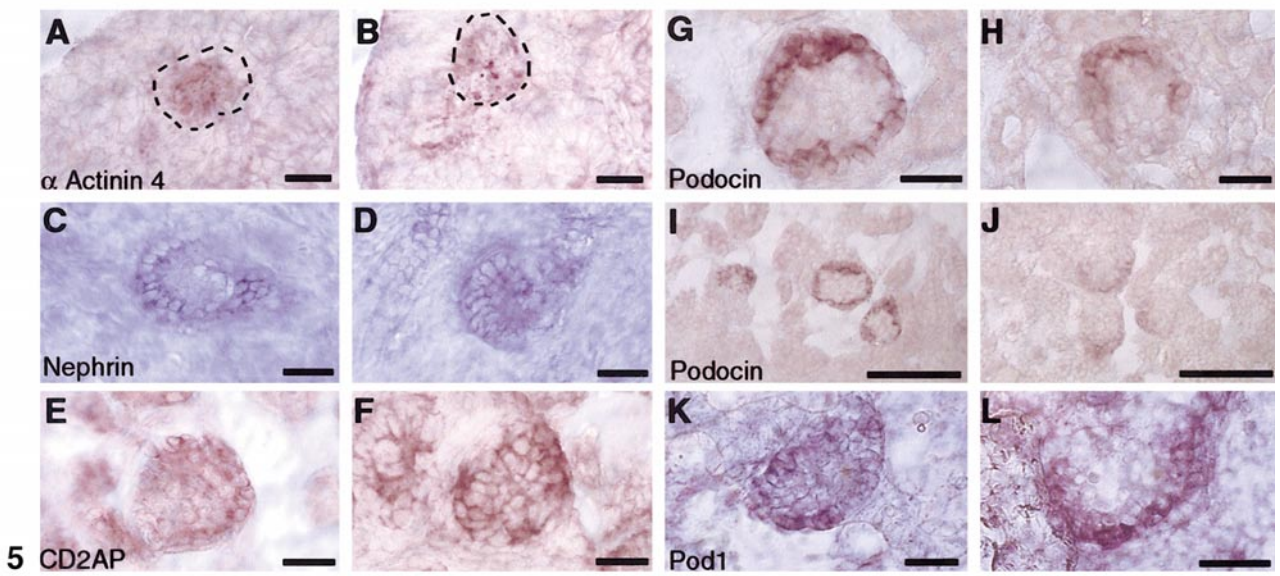


5 CD2AP Pod1

FIG. 4. Kreisler protein is present in kr^{enu} homozygotes, but absent in $Pod1^{-/-}$ kidneys. Immunofluorescence detects Kreisler protein in the nuclei of podocytes of +/+ (A) and kr^{enu}/kr^{enu} (B) mutant neonatal kidneys at the capillary loop stage. Immunofluorescence with an anti-Integrin $\alpha 3$ antibody detects equivalent expression in the podocytes of normal (+/+) (C) and kr^{enu}/kr^{enu} (D) neonatal kidneys. Laminin $\beta 2$ is present in the GBM of normal (+/+) (E) and kr^{enu}/kr^{enu} (F) glomeruli. PCNA cannot be detected in podocytes of +/+ (G) and kr^{enu}/kr^{enu} (H) neonatal kidneys at the capillary loop stage. Autofluorescence of erythrocytes is seen in both (G) and (H). (I) The DAPI counter-stained +/+ glomerulus seen in (G). (J) The DAPI counter-stained kr^{enu}/kr^{enu} glomerulus seen in (H) is shown. (L) Autofluorescence of erythrocytes can be seen in the absence of anti-PCNA antibody, as shown here in a +/+ glomerulus. The DAPI counter-stained glomerulus for (L) is shown in (K). Scale bars represent 20 μm. Each pair of panels is shown at the same magnification in normal and mutant animals.

FIG. 5. Expression of podocyte-specific genes in normal and mutant kidneys. Digoxigenin RNA *in situ* hybridization experiments were performed on cryosections from +/+ (A, C, E, G, K) and kr^{enu}/kr^{enu} (B, D, F, H, L) neonatal kidneys. *α-Actinin 4* is expressed in normal (A) and mutant (B) animals starting at the S-shaped stage. S-shaped-stage glomeruli are outlined with a dashed line. *Nephrin* is expressed at equivalent levels in podocytes of normal (C) and kr^{enu}/kr^{enu} (D) neonatal kidneys. CD2AP is expressed in both +/+ (E) and kr^{enu}/kr^{enu} (F) podocytes. *Podocin* expression appears slightly lower in kr^{enu}/kr^{enu} (H, J) podocytes than in normal (G, I) podocytes. *Pod1* is expressed equivalently in +/+ (K) and kr^{enu}/kr^{enu} (L) neonatal kidneys. Experiments were performed on 15.5-dpc and neonatal kidneys. Neonatal kidneys are shown here. All experiments were performed in triplicate on independent +/+, $+/kr^{enu}$, and kr^{enu}/kr^{enu} kidneys. Scale bars represent 20 μm in all panels except for (I) and (J). In (I) and (J), the scale bars represent 100 μm.

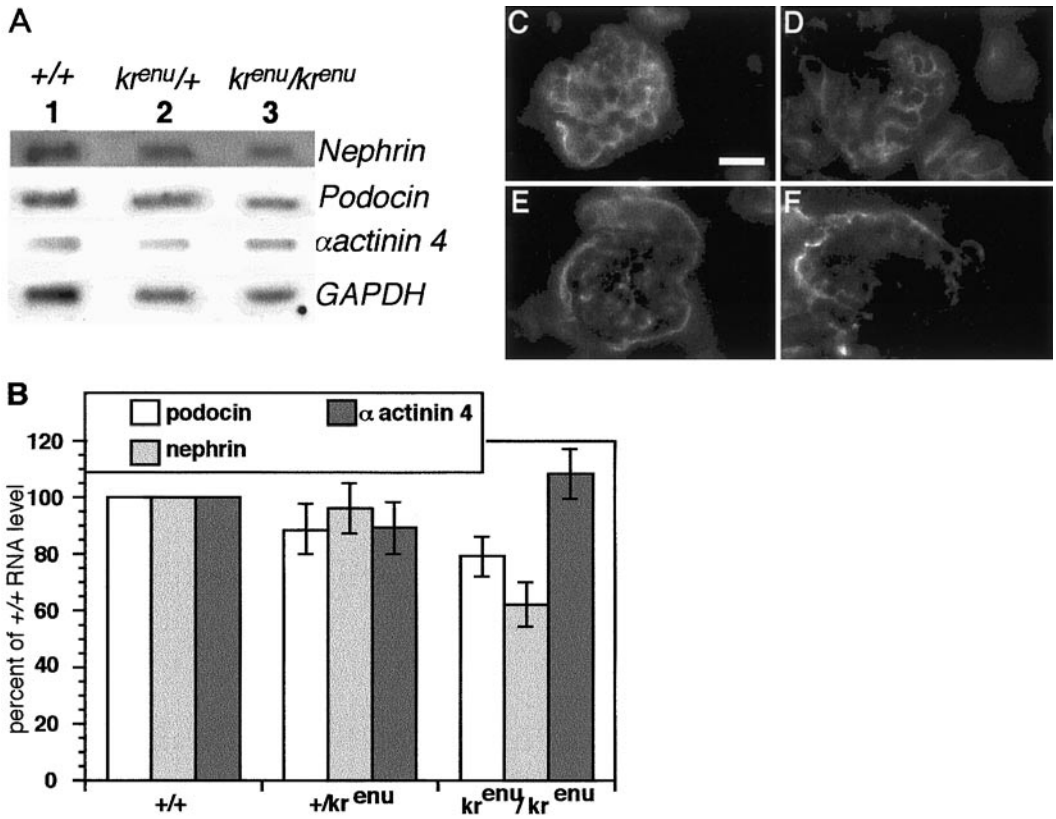


FIG. 6. Nephrin and Podocin are expressed at slightly lower levels in neonatal kr^{enu}/kr^{enu} kidneys. (A) Levels of mRNA of murine homologues of kidney disease genes were examined by RNA slot blot analysis. Each well contains 10 μg of total RNA from the following tissues: lane 1, neonatal +/+ kidney; lane 2, neonatal $kr^{enu}/+$ kidney; and lane 3, kr^{enu}/kr^{enu} neonatal kidney. *Nephrin*, *Podocin*, and α *Actinin 4* are all expressed in normal, heterozygous, and homozygous kr^{enu} mice. (B) Densitometry was used to quantify RNA levels. Levels of *Podocin* and *Nephrin* were slightly lower in kidneys from kr^{enu}/kr^{enu} animals relative to their +/+ and $kr^{enu}/+$ littermates. Levels of α *Actinin 4* are approximately equivalent in normal and mutant kidneys. (C–F) Localization of Podocin and Nephrin proteins was examined by immunofluorescence. Podocin protein is present in podocyte membranes from normal (+/+) (C) and kr^{enu}/kr^{enu} (D) kidneys as detected by immunofluorescence with an anti-Podocin antibody. Nephrin is localized to the membranes of normal (E) and kr^{enu}/kr^{enu} (F) podocytes as detected with immunofluorescence with an anti-Nephrin antibody. The scale bar represents 20 μm. All panels are shown at the same magnification.

isler protein at equivalent levels in normal and mutant podocytes at the capillary loop stage. Thus, the kr^{enu} mutation does not destabilize Kreisler protein.

The basal membrane of podocyte foot processes (the "sole") attaches to the extracellular matrix of the GBM (Abrahamson, 1991). Numerous proteins, including Integrin $\alpha 3\beta 1$, are localized to the "soles" of podocyte foot processes and are essential for the maturation of podocytes. Integrin α3-deficient mice, for instance, do not form foot processes (Kreidberg *et al.*, 1996). In kr^{enu}/kr^{enu} podocytes, Integrin α3 is expressed and localized to the cell membrane much as in normal podocytes as detected by immunofluorescence with anti-Integrin α3 antibody (Figs. 4C and 4D).

The "soles" of the podocyte foot processes attach to the GBM, and defects in the GBM can cause podocyte foot process effacement. *In vivo* labeling and ultrastructural studies indicate that dual membranes produced by the podocyte and the glomerular endothelial cells fuse to form the GBM during the capillary loop stage. In mice lacking *Collagen IV* or *Laminin* α*5*, defects in the ultrastructural appearance of the GBM coincide with the fusion and loss of podocyte foot processes (Cosgrove *et al.*, 1996; Lu *et al.*, 1999; Miner and Li, 2000). In the absence of some GBM components, such as Laminin α5, an intact GBM is never formed, and hence, podocyte foot processes can never attach to the GBM. Other molecules, such as Collagen COL4A3, and Laminin β2, appear to be required not for the initial formation of the GBM, but for its maintenance. For example, at birth, both the GBM and podocyte foot processes appear normal in mice lacking either Col4A3 or Laminin β2. GBM defects and pedicle loss only become apparent at 4 weeks of age in mice with loss-of-function

mutations in collagen COL4A3, a mouse model for human autosomal Alport syndrome, and at 2 weeks in mice lacking Laminin β2 (Cosgrove *et al.,* 1996; Noakes *et al.,* 1995).

In kr^{enu}/kr^{enu} kidneys, the GBM appears normal when examined by transmission electron microscopy (Figs. 3G and 3H). Notably, at present, only in mice lacking Laminin β2 is foot process effacement observed prior to any visible ultrastructural abnormalities in the GBM. In all other known "GBM" mutants, foot processes appear normal as long as the GBM appears ultrastructurally intact, as it does in kr^{enu}/kr^{enu} kidneys. Laminin β2, which is expressed starting at the capillary loop stage, can be detected in the GBM of both normal and mutant glomeruli by immunofluorescence (Figs. 4E and 4F). Current analyses of mouse mutants in GBM components have not identified any single mutation with a phenotype identical to that observed in kr^{enu}/kr^{enu} glomeruli.

Previously, disruption of the cMaf gene has been shown to arrest differentiation of lens fiber cells. The absence of cMaf protein results in the failure of lens fiber cells to withdraw from the cell cycle (Ring *et al.,* 2000). In kr^{enu} homozygotes, we did not detect mitotic figures in any podocytes examined by transmission electron microscopy. Furthermore PCNA is expressed in actively dividing cells, but cannot be detected in podocytes from either normal or kr^{enu}/kr^{enu} neonates by immunofluorescence (Figs 4G–4J). Thus, in contrast to lens fiber cells in cMaf knock-outs, kr^{enu}/kr^{enu} podocytes do not re-enter the cell cycle inappropriately. These observations suggest that the kr^{enu} mutation disrupts the ability of Kreisler to transcribe molecules required specifically for podocyte differentiation, but does not affect any potential role that the Kreisler protein may play in cell cycle withdrawal during podocyte differentiation.

Expression of Murine Orthologues of Human Kidney Disease Genes in kr^{enu}/kr^{enu} Podocytes

Recently, advances in human genetic kidney diseases have identified additional molecules required for podocyte function and the formation or maintenance of pedicles. Mutations in *ACTN4*, the gene encoding *α-Actinin 4*, cause dominant familial focal segmental glomerulosclerosis (FSGS) (Kaplan *et al.,* 2000). *α-Actinin 4* is an actin filament cross-linking protein thought to regulate the actin cytoskeleton of podocytes. *In vitro* the dominant mutations identified in familial FSGS bind filamentous actin more tightly than normal α-Actinin 4 protein does. It is not known whether *α-Actinin 4* is required to maintain foot processes and the slit diaphragm or whether increased rigidity of the foot processes might make them more vulnerable to damage. Expression of *α-Actinin 4* commences in podocytes of the late S-shaped body and early capillary loop stages well before that of *Kreisler*. We examined expression of murine *α-Actinin 4* in kr^{enu}/kr^{enu} kidneys by RNA *in situ* hybridization and RNA slot blot analyses. We found no difference in the temporal and spatial expression nor in the level of expression of *α-Actinin 4* between normal and kr^{enu}/kr^{enu} kidneys (Figs. 5A, 5B, and 6A). Thus, although it is possible that *Kreisler* acts in parallel with the genes that regulate *α-Actinin 4*, we believe that *Kreisler* acts at a slightly later stage, and in the future may serve as a marker for the mid-capillary loop to mature stages.

A highly unique feature of podocyte foot processes is the presence of the "slit diaphragm." The slit diaphragm is inserted between foot processes just above their "sole" and thus joins podocyte foot processes laterally. A complex that contains three known proteins, Nephrin, Podocin, and CD2 adapter protein, is required for slit diaphragm function and assembly. *Nephrin*, the gene mutated in congenital nephrotic syndrome of the Finnish type (NPHS1), encodes a transmembrane protein of the Immunoglobulin superfamily that is localized at the slit diaphragm joining the interdigitated pedicles (Kestila *et al.,* 1998). Mutations in *Nephrin* cause severe proteinuria and usually lead to death within the first 2 years of human life (Donoviel *et al.,* 2001; Kestila *et al.,* 1998). *Nephrin* expression in humans and mice has been detected in podocytes of the capillary loop stage glomeruli, while in rats, *Nephrin* mRNA is already present in S-shaped bodies (Kawachi *et al.,* 2000; Putaala *et al.,* 2000). In addition, in the mouse, CD2 adapter protein (CD2AP) has been identified as another component of the slit diaphragm. CD2AP is a Src homology 3 domain-containing protein that interacts directly with Nephrin and is required for assembly of the slit diaphragm in the mouse (Lehtonen *et al.,* 2000; Li *et al.,* 2000). In mutant mice lacking CD2AP, podocyte foot processes form initially, but begin to efface starting 1 week after birth. *Podocin* was identified as the gene mutated in autosomal recessive steroid-resistant nephrotic syndrome (NPHS2) on chromosome 1q25–31 and is first expressed at the capillary loop stage. The Podocin protein is structurally related to the integral membrane phosphoprotein stomatin.

We examined expression of these components of the slit diaphragm in +/+, $+/kr^{enu}$, and kr^{enu}/kr^{enu} kidneys by RNA *in situ* hybridization and RNA slot blot analyses. We found no difference in the temporal and spatial expression of *Nephrin*, *Podocin*, or *CD2AP* between normal and kr^{enu}/kr^{enu} kidneys (Figs. 5C–5J). Although the level of *Podocin* expression was indistinguishable in the cranial ganglia of normal, heterozygous, and homozygous mutant embryos at 15.5 dpc (data not shown), *Podocin* expression consistently appeared lower in kr^{enu}/kr^{enu} kidneys. In RNA *in situ* hybridization experiments, *Podocin* expression can normally be detected after 6–8 h of color development in neonatal kidneys, but could be detected only upon 12–18 h of color development in RNA *in situ* hybridization in mutant kidneys, and even then the observed level appears reduced (Figs. 5G–5J). We examined mRNA levels of *Podocin*, *Nephrin*, and *α Actinin 4* in RNA slot blots to determine whether these were affected in kr^{enu}/kr^{enu} mutants (Figs. 6A and 6B). The levels of *Nephrin* and *Podocin* mRNA appear slightly reduced in kr^{enu}/kr^{enu} kidneys. When we quantified the levels of *Nephrin* and *Podocin* mRNA by

densitometry, *Podocin* and *Nephrin* mRNA levels are reduced to 76 ± 7 and 62 ± 9%, respectively, in kr^{enu} homozygotes relative to their heterozygous and +/+ littermates. Levels of *α Actinin 4* RNA in neonatal kidneys appear equivalent between kr^{enu} homozygotes, heterozygotes, and unaffected littermates. By immunofluorescence with anti-Podocin and anti-Nephrin antibodies, we determined that Podocin and Nephrin proteins are localized to the membranes of both normal and kr^{enu}/kr^{enu} podocytes (Figs. 6C–6E). Thus, transcription of *Podocin* and *Nephrin* does not depend on active Kreisler homodimers, but indirectly or directly, Kreisler may have a small effect on the transcription or stability of *Podocin* and *Nephrin* mRNAs. In the future, identification of direct Kreisler targets during podocyte differentiation may reveal additional proteins important in glomerular function and possibly in kidney disease.

Kreisler Acts Downstream of the Pod1 Transcription Factor

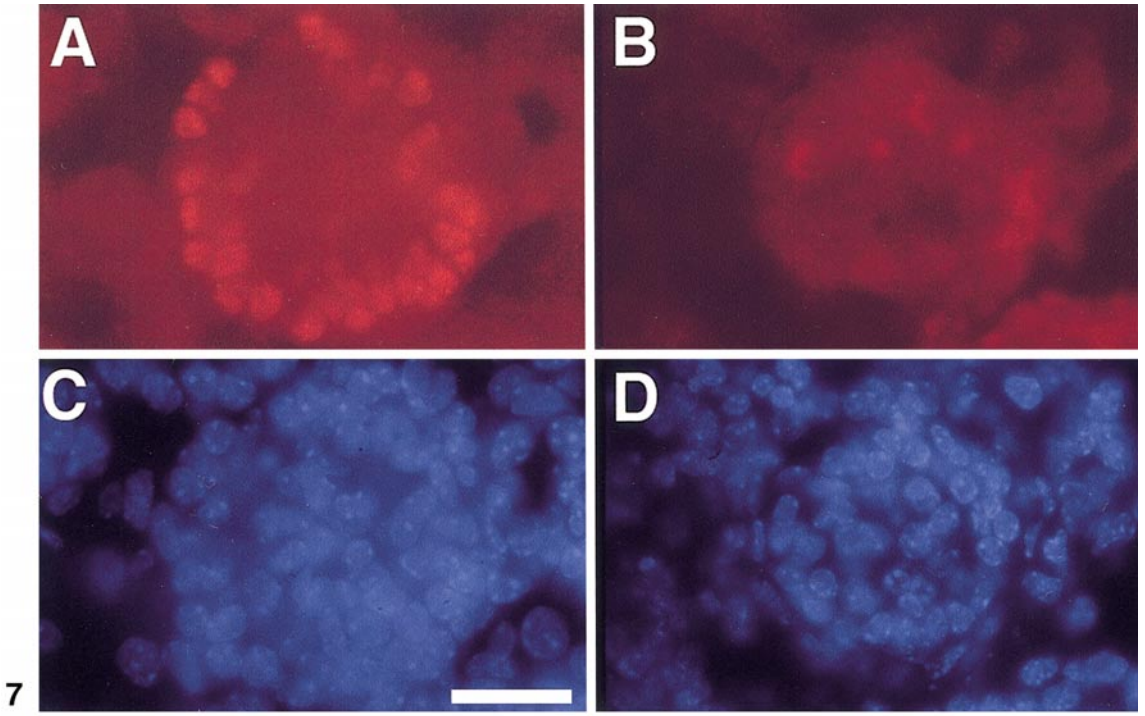
To begin to dissect the transcriptional hierarchy that governs podocyte differentiation, we examined the expression of *Kreisler* in mice homozygous for a mutation in the basic helix–loop–helix transcription factor Pod1 (epicardin/capsulin). *Pod1* is expressed in mesenchymal tissues of the developing kidney and in podocytes (Hidai *et al.*, 1998; Lu *et al.*, 1998; Quaggin *et al.*, 1998; Robb *et al.*, 1998). Disruption of *Pod1* causes podocytes to remain columnar shaped and to appear as though they are arrested in the S-shaped stage. A few rare primitive foot processes can be detected (Quaggin *et al.*, 1999). This phenotype is significantly more severe than that observed in kr^{enu}/kr^{enu} kidneys, where overall glomerular differentiation appears to be unaffected and podocytes do not appear columnar-shaped, but are simply not developing foot processes. At present, it is unclear whether the more severe phenotype in *Pod1* mutants is a consequence of the podocyte-specific role of *Pod1* or because of a defect in mesenchymal cells that surround the glomerulus (Quaggin *et al.*, 1998). In kr^{enu}/kr^{enu} animals, *Pod1* was expressed normally in the podocytes of 15.5-dpc and neonatal kidneys (Figs. 5K and 5L). However, when we examined glomeruli from $Pod1^{-/-}$ mutant mice, we could not detect appreciable amounts of Kreisler protein in the podocytes (Fig. 7). Taken together, these observations suggest that *Pod1* acts upstream of *Kreisler* in podocyte differentiation and that *Pod1* may have a podocyte-specific cell-autonomous role.

DISCUSSION

Aside from *Kreisler,* a variety of other transcription factors play important roles in glomerular development. All of these appear to act at earlier stages of kidney development or podocyte differentiation than *Kreisler* does. Genetic experiments have implicated the Wilm's tumor suppressor gene, *WT1*, in podocyte development (Dressler *et al.*, 1993). Patients with Denys–Drash syndrome, which is associated with specific heterozygous mutations of *WT1*, are not only at increased risk for developing Wilm's tumor, but also develop a nephropathy associated with grossly abnormal podocyte morphology (Pelletier *et al.*, 1991). Mice homozygous for a null allele of *WT1* fail to develop kidneys; therefore, a possible later role of *WT1* in podocyte development has not yet been assessed (Kreidberg *et al.*, 1993). WT1 protein is detected in podocytes of the early comma-shaped glomerulus well in advance of *Kreisler* expression and is maintained throughout podocyte development and in mature podocytes. Our results would predict that the dedifferentiated tumorigenic state in Wilm's tumors and in Denys–Drash syndrome may be associated with down-regulation of *Kreisler*. Assays of *Kreisler* expression might, in the future, serve as prognosticators of tumor progression. In contrast to humans, no increased occurrence of renal tumors is observed in mice heterozygous for a *WT1* null mutation, and similarly we have not observed tumors or cysts in kidneys from adult kr^{enu} heterozygotes.

As a bZip protein, Kreisler acts by forming complexes with other transcription factors and other bZip proteins in particular. At present, the only other bZip protein with a known role in kidney development is *EYA1*, a mammalian homologue of the *eyes absent* (*eya*) gene from *Drosophila melanogaster* (Abdelhak *et al.*, 1997). In humans, mutations in *EYA1* are responsible for the autosomal dominant branchio-oto-renal (BOR) syndrome, which is characterized by varying combinations of branchial, outer, middle, and inner ear, and renal anomalies. In the mouse, $Eya1^{-/+}$ animals show renal abnormalities and a conductive hearing loss equivalent to human BOR syndrome, while $Eya1^{-/-}$ mice lack ears and kidneys due to defective inductive tissue interactions and apoptotic regression of the organ primordia (Johnson *et al.*, 1999; Xu *et al.*, 1999). *Eya1* expression is observed in the condensing mesenchymal cells of the kidney and consistent with the excretory and collecting system anomalies of BOR syndrome. We and others have not detected expression of *EYA1* in mouse podocytes (data not shown), nor have specific podocyte defects been reported in BOR syndrome patients (Kalatzis *et al.*, 1998). This lack of coexpression in podocytes precludes a biologically relevant interaction during glomerular development, and thus, Kreisler is likely to interact with other as yet unidentified proteins to regulate podocyte specific transcription.

Mutations in *Lmx1b*, which in humans are responsible for nail patella syndrome, cause thickening and gaps in the GBM and an apparent lack of foot processes (Chen *et al.*, 1998; Dreyer *et al.*, 1998). Patients with nail patella syndrome, which is localized on chromosome 9q34, have a significantly thickened GBM, and renal insufficiency with proteinuria occurs in 30% of all patients. *Lmx1b* expression commences in the S-shaped body, and thus, prior to that of *Kreisler*. In the roof plate of the neural tube, *Kreisler* is downstream of *Lmx1a*, which is mutated in the classical mouse *dreher* mutation (Millonig *et al.*, 2000). Taken



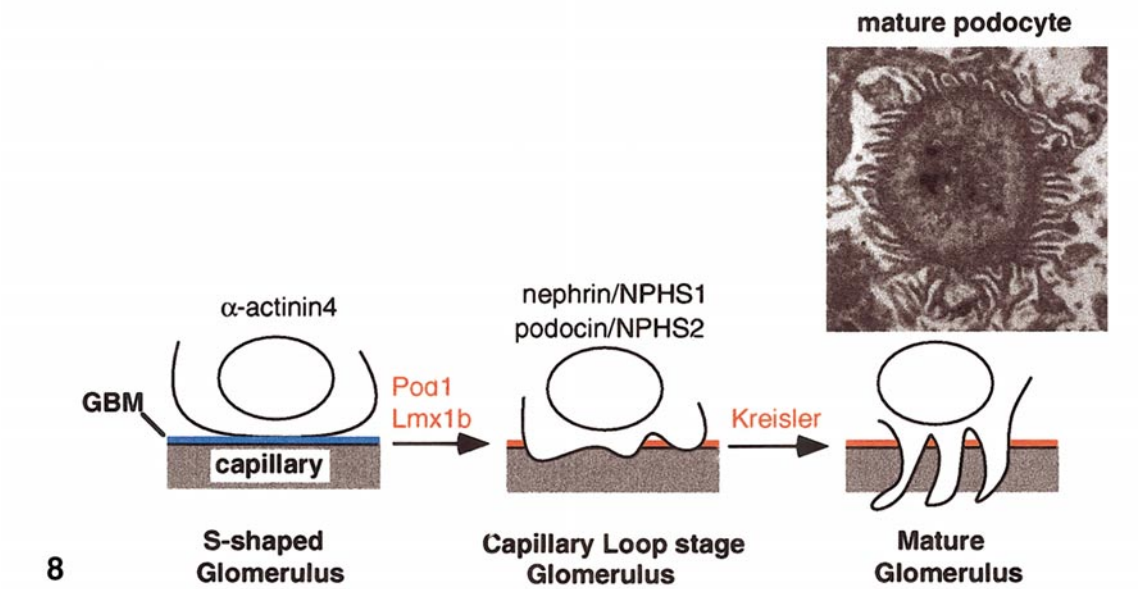


FIG. 7. Kreisler protein is absent in kidneys in the podocytes of *Pod1*$^{-/-}$ mice. While Kreisler protein is present in podocytes from mice heterozygous for a mutation in *Pod1* (A, C), it cannot be detected in podocytes from *Pod1*$^{-/-}$ mice (B, D) by immunofluorescence with anti-Kreisler antibody. All panels are shown at the same magnification. The scale bar represents 20 μm. Normal (C) and *kr*^{enu}/*kr*^{enu} (D) glomeruli that are shown in (A) and (B), respectively, were counter-stained with DAPI.

FIG. 8. Schematic diagram of podocyte differentiation. At the S-shaped stage of glomerular development, podocytes appear cuboidal and in their appearance resemble those observed in *Pod1*$^{-/-}$ mice. At this stage, *Pod1* and *Lmx1b* are expressed. *α-Actinin 4* expression is also seen at this stage. During the transition from the S-shaped to the capillary loop stage, the composition of the GBM changes. *Lmx1b* regulates the expression of some of the components of the GBM. At the capillary loop stage, the GBM has fused, for the most part, into one thick membrane. During maturation of the podocyte, the foot processes form on the outer GBM, until the GBM is completely fringed by foot processes. During the capillary loop stage, podocytes begin to express *Nephrin*, *Podocin*, and *CD2AP*. In kidneys from mice homozygous for the *kr*^{enu} mutation, foot processes cannot form because of a defect in podocyte differentiation. However, the GBM appears electron-microscopically normal and *Nephrin*, *Podocin*, *CD2AP*, and *α-actinin 4* are expressed in these animals. The severity of the defect, the appearance of the podocytes, and the time of expression all suggest that *Kreisler* acts directly or indirectly downstream of *Pod1* and *Lmx1b*, and indeed Kreisler protein can no longer be detected at normal levels in podocytes from *Pod1*$^{-/-}$ mice. Electron microscopic cross-section through a mature podocyte shows the foot processes extending from the podocyte cell body. Pc, podocyte (blue); c, capillary; m, mesangial cell (solid black); blue denotes early GBM, which differs in its composition from the fused glomerular basement membrane in the capillary loop stage, shown in red.

together, these observations suggest that *Kreisler* acts at a later stage of podocyte differentiation and may be downstream, possibly directly, of *Lmx1b*. It is quite possible that some of the roof plate-specific and podocyte-specific regulatory elements of Kreisler may overlap.

Our analyses have revealed that *Kreisler* acts downstream of the Pod1 bHLH transcription factor. In $Pod1^{-/-}$ mice, glomerular maturation is arrested well before the podocyte-specific expression of Kreisler would normally commence. In $Pod1^{-/-}$ mice, the podocytes appear columnar-shaped and no foot processes form (Quaggin *et al.*, 1999). In kr^{enu} homozygotes, the morphology of podocytes appears normal, but foot processes do not extend. Taken together, these observations suggest that Kreisler is more likely to be an indirect rather than a direct target of the Pod1 transcription factor. Furthermore, *Kreisler* is required for the final stage in podocyte differentiation, the progression through the capillary loop stage to the mature glomerulus. Thus, Kreisler regulates podocyte differentiation during the capillary loop stage and governs such processes as either the extension or attachment of the foot processes or possibly the assembly of the filtration apparatus.

While we could assess the role of Kreisler in initial podocyte differentiation, we could not analyze its role in maintenance of functional podocytes or in recovery from podocyte damage. For example, in neonatal kidneys, *Kreisler* is not required for the initial production or fusion of the GBM, and the GBM appears ultrastructurally normal. However, *Kreisler* may play a role in the later podocyte-specific generation of GBM in mature podocytes. Such a role would only be uncovered during maturation and aging of the animals. In that case, inactivation of Kreisler in adult mice might cause severe nephrotic syndrome within 4–8 weeks or prevent animals from recovering after short-term podocyte insults. Thus, conditional or inducible kidney-specific alleles of *Kreisler* would be invaluable for further understanding of podocyte development and renal disease.

The levels of two known components of the filtration apparatus, *Nephrin* or *Podocin*, may be slightly reduced in kr^{enu} homozygotes, but these genes are not likely to be direct targets of Kreisler. This is especially true of *Nephrin*, which has been detected in other animals in podocytes of the S-shaped stage, and thus before the appearance of *Kreisler* expression. The slightly reduced expression of *Podocin* and *Nephrin* may reflect the reduced overall membrane surface area of podocytes arrested at the capillary loop stage. Thus, a dynamic feedback loop that senses the surface area of the membrane may help regulate the amount of these proteins.

The podocyte-specific target genes of Kreisler remain to be identified. We expect most of these to be distinct from those regulated by Kreisler in the hindbrain. For example, we could not detect expression of *Hoxa3* or *Hoxb3*, two known Kreisler target genes in the hindbrain, in podocytes at any stage of glomerular development (S.P.C., unpublished observations; Mansour *et al.*, 1988; Manzanares *et al.*, 1997). Conversely, we and others have not detected expression of podocyte-specific genes in the fifth and sixth rhombomeres of the embryonic hindbrain. The list of known genes with podocyte-specific expression patterns has been expanding rapidly, and testing each known podocyte-specific gene for its expression in kr^{enu} mutants is rapidly becoming a daunting task. Hence, podocyte-specific targets of Kreisler may perhaps best be identified in future subtractive hybridization or microarray experiments. Overall, our results suggest that such experiments would identify additional components important for podocyte function and in human kidney disease.

ACKNOWLEDGMENTS

We thank Ralph Dileone for advice on *in situ* RNA hybridization on sections, Claire Antignac for the *Podocin* probe, Michael DiPersio for the anti-Integrin α3 antibody, Larry Holzman for the anti-Nephrin antibody, Peter Mundel for the anti-Podocin antibody, Rupert Timpl and Joshua Sanes for anti-Laminin β2 antibodies, and Jeff Miner for suggestions. This work has been supported by Canadian CIHR Grant #MT-14312 (to S.P.C.) S.E.Q. is a CIHR clinician scientist and a Canadian Foundation of Innovation young investigator. G.S.B. is an associate investigator of the Howard Hughes Medical Institute. S.P.C. is an MRC/CIHR scholar, a Canadian Foundation of Innovation young investigator, and an EJLB foundation scholar.

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Received for publication November 11, 2001
Revised June 10, 2002
Accepted June 10, 2002
Published online July 31, 2002